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Exploration of Binding and Toxic Site of Botulinum Neurotoxin.

Final Report

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The research was directed to identify and characterize the three active regions of the large (M_r 150,000) protein, botulinum neurotoxin, responsible for i) binding to the receptors, ii) forming channels on membrane, and iii) blocking acetylcholine release. The heavy chain (M_r 100,000) of the neurotoxin first binds to the specific sites (receptors) on the nerve terminals. This binding is necessary for the light chain (M_r 50,000) to be fixed at these specific sites. Then the light chain induces paralysis. The heavy chain, but not the light chain, forms channels in planar bilayer membranes. These channels have pH and voltage dependent gating properties. Planter nerves-lumbrical of the hind paw of the mouse were introduced as neuromuscular preparation for studying the neurotoxin induced paralysis, particularly for electron microscopy of the binding of radiolabelled neurotoxin.

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FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Summary

The well known pharmacological action of botulinum neurotoxin (NT) is on the neuromuscular junctions; release of acetylcholine is blocked and consequently flaccid muscle paralysis occurs. The mechanism of blockade of acetylcholine release at the molecular level is not known. But it is generally agreed that the NT appears to act in three steps. First it binds to the nerve membrane (= binding step); than it (or possibly a fragment) moves to a new site presumably inside the axon (= translocation or internalization step), and finally the NT blocks acetylcholine release (= the toxic or paralytic step). The research studies, under the contract, were directed to identify and characterize the three active moieties of the NT responsible for i) binding to the host receptors, ii) forming channels on membrane, and iii) blocking acetylcholine release.

Body of the report (Text)

1. Role of the heavy and light chains in the neuroparalysis:

Botulinum NT when fully active, is a dichain protein composed of a heavy and a light chain (mol. wt. ~100,000 and ~50,000, respectively) that are held together by non-covalent bonds and at least one disulfide bond. The NT is synthesized by Clostridium botulinum in any of seven antigenically distinct forms, called types A-G. The paralysis of neuromuscular preparations (mouse phrenic-nerve hemidiaphragm) induced by the dichain NT was delayed (antagonized) if the neuromuscular preparations were incubated with the isolated and purified heavy chain prior to or during incubation with the parent dichain NT. Neuromuscular preparations became paralyzed when incubated with the heavy chain, then washed free of nonbound heavy chain, and then further incubated with the light chain. The paralysis did not occur if the neuromuscular preparation was incubated first with the light and then with the heavy chain. These observations make a persuasive case that the heavy chain first binds to the specific sites (receptors) on the nerve terminals. This binding is necessary for the light chain to be fixed at these specific sites. Then the light chain (or combinations of the light and heavy chain) in some way induces paralysis through a mechanism very similar to that of the parent dichain NT. This general model of the structure-function relationship in the mode of action of botulinum NT was developed with types A and B NT, rather than one NT type. Results using the two types are consistent. This is the first direct experimental demonstration of the role of the two subunit chains of the NT (see publication #3 and 5).

2. Channels formed by the NT in planar lipid bilayers:

Pure preparations of heavy chain (mol. wt. 102,000) derived from type B dichain NT (mol. wt. 152,000) formed channels in planar bilayer membranes. These channels have pH dependent and voltage dependent gating properties similar to channels formed by the heavy chain from diphtheria toxin and the heavy chain from tetanus toxin. The light chain derived from the type B NT and the intact single chain NT under these conditions were devoid of channel forming activity. Selectivity experiments with anions and cations show that the channels formed are large; large enough to serve as "tunnel proteins" for translocation of active fragments (see publication #1).

3. Introduction of lumbrical muscle preparation for the study of botulinum NT:

The lumbricals, to our knowledge, have not been used to study the mechanism of action of the NT. The dissection of the hind paws of a mouse provide at least 4, and sometimes as many as 8 nerve muscle preparations that are thin, short, and flat. The location of neuromuscular junctions (NMJ) is highly predictable, hence well suited for observation with Nomarski interference phase contrast optics and for electron microscopy (e.m.), and also for electrophysiology. The muscles have comparatively few fibers hence their response to experimental agents such as botulinum NT is rapid and complete. The lumbrical preparations incubated with various concentrations of type A NT were paralyzed in NT concentration-dependent fashion. The tissues

also responded to type E NT trypsinized and not trypsinized demonstrating the expected over 100-fold activation of the NT. The muscle paralysis (twitch), cessation of end plate potentials and miniature end plate potentials following exposure to the NT is qualitatively similar to the reference mouse phrenic nerve hemidiaphragm preparations. The lumbricals are 2 fibers thick compared to the 5 fiber thickness of the hemidiaphragm. We believe that the lumbrical muscles of mouse would be better than the hemidiaphragm as NMJ preparation for our planned e.m. work on the binding of the NT with cholinergic presynaptic membranes (see publication #2 and 4).

Publications supported by contract DAMD 17-83-C-3034

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